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AUTHOR(S):

Kawamoto, Yasuhiro; Ito, Hidefumi; Ihara, Masafumi; Takahashi, Ryosuke

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# **Immunohistochemical localization of XIAP in brainstem-type and cortical Lewy bodies**

Running head: XIAP immunoreactivity in Lewy bodies

Yasuhiro Kawamoto<sup>a,b</sup>, Hidefumi Ito<sup>a</sup>, Masafumi Ihara<sup>a</sup> and Ryosuke Takahashi<sup>a</sup>

<sup>a</sup>Department of Neurology, Faculty of Medicine, Kyoto University, Kyoto, Japan and

<sup>b</sup>Department of Neurology, Seijinkai Rakusaishimizu Hospital, Kyoto, Japan

Correspondence to Yasuhiro Kawamoto, MD, PhD, Department of Neurology, Faculty of Medicine, Kyoto University, 54 Shogoin-Kawaharacho, Sakyo-ku, Kyoto 606-8507, Japan

Tel: + 81 75 751 3766; fax: + 81 75 751 3265;

e-mail: [kawamoto@kuhp.kyoto-u.ac.jp](mailto:kawamoto@kuhp.kyoto-u.ac.jp)

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**XIAP blocks the apoptosis by binding to and inhibiting caspases-3, -7 and -9. XIAP is negatively regulated by the mitochondrial serine protease HtrA2/Omi. The aim of this study was to investigate the role of XIAP and the relationship between XIAP and HtrA2/Omi in patients with Parkinson's disease or dementia with Lewy bodies. We performed immunohistochemical studies on XIAP in formalin-fixed, paraffin-embedded sections from 8 normal subjects, 10 patients with Parkinson's disease, 5 patients with dementia with Lewy bodies and 7 patients with Alzheimer's disease, and then double-labeling immunohistochemistry for XIAP and HtrA2/Omi in sections from the Parkinson's disease and dementia with Lewy bodies cases. Brainstem-type and cortical Lewy bodies were intensely immunostained for XIAP, and XIAP immunoreactivity was localized to the halos of brainstem-type Lewy bodies and to the entire bodies of cortical Lewy bodies. Double immunofluorescence analyses showed that XIAP and HtrA2/Omi immunoreactivities were co-localized to both types of Lewy bodies. Our results suggest that XIAP may be partially associated with the pathogenesis of Parkinson's disease and dementia with Lewy bodies.**

## Introduction

The inhibitor of apoptosis protein (IAP) family members have been widely conserved in a large number of eukaryotic species, ranging from insects to humans, and play an important role in regulating apoptotic cell death [1]. The IAP family of proteins is characterized by a novel domain of about 70 amino acids termed the baculovirus IAP repeat (BIR), the name of which derives from the original discovery of IAP family proteins in baculoviruses [1,2]. X-linked IAP (XIAP), a member of the IAP family, has the ability to directly bind to and inhibit the initiator caspase-9, and the effectors caspase-3 and caspase-7, thus blocking the apoptotic process [3,4]. XIAP contains three tandem BIR domains [1,2,4], and the caspase-3 and -7 inhibitory functions of XIAP were demonstrated to be localized to the second BIR domain [5], whereas the third BIR domain of XIAP was shown to be sufficient for inhibiting caspase-9 activity [6]. These data suggest that the three BIR domains within XIAP may have distinct functions, and that XIAP may inhibit the initiator and effector caspases in a different manner.

XIAP is negatively regulated by several proteins, including the mitochondrial serine protease HtrA2/Omi [7-10], which is released from the mitochondria into the cytosol upon receiving various apoptotic stimuli [7-10]. HtrA2/Omi binds to XIAP and blocks

its caspase-inhibitory activities [7-10]. Recently, we reported the immunohistochemical localization of HtrA2/Omi in brainstem-type and cortical Lewy bodies from brains with Parkinson's disease or dementia with Lewy bodies [11]. In the present study, we performed immunohistochemical studies on XIAP and HtrA2/Omi using autopsied brains with Parkinson's disease or dementia with Lewy bodies, and found that brainstem-type and cortical Lewy bodies were intensely immunostained for XIAP, and that the XIAP and HtrA2/Omi immunoreactivities were co-localized to both types of Lewy bodies.

## **Materials and methods**

### **Tissue preparation**

We studied autopsied brains from 8 control subjects without any neurological abnormalities (age range, 54-78 years; mean, 68.4 years; 6 men and 2 women), 10 patients with Parkinson's disease (age range, 66-90 years; mean, 77.5 years; 6 men and 4 women) and 5 patients with dementia with Lewy bodies (age range, 69-86 years; mean, 74.8 years; 4 men and 1 woman). Autopsied brains from 7 patients with Alzheimer's disease (age range, 67-89 years; mean, 78.4 years; 2 men and 5 women) served as disease controls. All brains were fixed in 10% neutral formalin for about 2

weeks at room temperature. Several paraffin-embedded tissue blocks, including the frontal and temporal cortices, midbrain and upper pons, were prepared and cut into 6- $\mu$ m-thick sections on a microtome. The paraffin-embedded sections were deparaffinized in xylene, and then followed by rehydration in a decreasing concentration of ethanol solutions. For routine pathological evaluation, deparaffinized sections from all cases were stained with the hematoxylin and eosin (H&E), Klüver-Barrera and modified Bielschowsky methods. The use of human materials was in agreement with the ethical guidelines set forth by Kyoto University.

### **Immunohistochemistry**

To examine the immunohistochemical localization of XIAP in normal and diseased brains, we used a goat polyclonal anti-XIAP antibody (AF8221, R&D systems, Minneapolis, MN, USA). The deparaffinized sections were pretreated with 0.3% hydrogen peroxide (Santoku, Tokyo, Japan) in 0.1 M phosphate-buffered saline (PBS) for 30 min at room temperature to inhibit endogenous peroxidase activity. After washing with 0.1 M PBS, the sections were blocked with 0.1 M PBS with 3% skim milk for 2 h at room temperature. After rinsing with 0.1 M PBS, the anti-XIAP antibody diluted in 0.1 M PBS (15  $\mu$ g/ml) was applied onto the sections, and the sections were incubated at

4°C overnight in a humidified chamber. After washing with 0.1 M PBS, the sections were reacted with a biotinylated anti-goat IgG (Vector Laboratories, Burlingame, CA, USA) diluted in 0.1 M PBS (1:200) for 1 h at room temperature, and then followed by incubation with an avidin-biotin-peroxidase complex (ABC) kit (Vector Laboratories) diluted in 0.1 M PBS (1:400) for 1 h at room temperature. After rinsing with 0.1 M PBS and then in 0.05 M Tris-HCl (pH 7.6), the sections were developed in a colorizing solution containing 0.02% diaminobenzidine tetrahydrochloride (Dojin, Kumamoto, Japan), 0.6% ammonium nickel (II) sulfate (Wako, Osaka, Japan) and 0.005% hydrogen peroxide in 0.05 M Tris-HCl (pH 7.6) for 10 min at room temperature. Some H&E-stained sections with brainstem-type or cortical Lewy bodies were color-photographed, decolorized with 70% ethanol, and were then immunostained with the anti-XIAP antibody using the ABC method described above. As a negative immunohistochemical control, some sections were incubated with normal goat serum, and no specific immunopositive staining was detected in these control sections (data not shown).

### **Double immunofluorescence staining**

To compare the anatomical distribution of XIAP-immunoreactive Lewy bodies to that of HtrA2/Omi-positive Lewy bodies in brains with Parkinson's disease or dementia with



Lewy bodies, we performed double-labeling immunohistochemistry using the anti-XIAP antibody and an anti-HtrA2/Omi antiserum, which was raised by immunizing rabbits with *E coli* expressing the C-terminal His<sub>6</sub>-tagged mature form of the human HtrA2/Omi protein [7]. Sections from all of the Parkinson's disease and dementia with Lewy bodies cases were incubated with a combination of the anti-XIAP antibody (15 µg/ml) plus the anti-HtrA2/Omi antiserum (1:200) in 0.1 M PBS at 4°C overnight. After washing with 0.01 M PBS, the sections were reacted with secondary antibodies consisting of a fluorescein isothiocyanate-conjugated swine anti-goat IgG (Biosource, Camarillo, CA, USA) and a tetramethylrhodamine-conjugated swine anti-rabbit IgG (DakoCytomation, Glostrup, Denmark). After rinsing with 0.01 M PBS, the slides were coverslipped with Vectashield (Vector Laboratories), and were then viewed with a fluorescence microscope system (BZ-9000, Keyence, Osaka, Japan).

### **Semiquantitative assessment of Lewy bodies immunostained for XIAP and HtrA2/Omi**

To evaluate the proportions of Lewy bodies which were immunoreactive for both XIAP and HtrA2/Omi, we prepared H&E-stained sections from all of the Parkinson's disease and dementia with Lewy bodies cases. After counting the number of brainstem-type

Lewy bodies in the substantia nigra and locus ceruleus in Parkinson's disease and the number of cortical Lewy bodies in the cingulate and parahippocampal cortices in dementia with Lewy bodies, these sections were decolorized with 70% ethanol, and were then double-immunostained for XIAP and HtrA2/Omi. After counting the number of double-immunolabeled Lewy bodies in the same areas, we calculated the percentage of double-immunolabeled Lewy bodies in each section, and then calculated the average percentages of brainstem-type Lewy bodies in the substantia nigra and locus ceruleus and the average percentages of cortical Lewy bodies in the cingulate and parahippocampal cortices.

## Results

### **XIAP immunoreactivity in normal and diseased brains**

In the frontal and temporal cortices from control subjects, both pyramidal and non-pyramidal neurons were immunostained for XIAP at various degrees of intensity (Fig. 1a). Granular XIAP immunoreactive products accumulated in the somata and proximal processes of some neurons (Fig. 1b). In the frontal and temporal white matter from the control subjects, XIAP-immunopositive glial cells were scattered throughout (Fig. 1c), some of which were oligodendrocytes by morphology (Fig. 1d). These neuronal and

glial immunolabeling patterns suggest that XIAP may be partially activated even in the normal brains.

In the substantia nigra and locus ceruleus from normal controls, about 60% of the pigmented neurons were mildly immunostained for XIAP, and the rest were moderately immunostained (Fig. 1e). In the same areas from patients with Parkinson's disease or dementia with Lewy bodies, a similar immunolabeling pattern was observed, and XIAP immunoreactivity was spared in the remaining neurons (Fig. 1f).

### **XIAP immunoreactivity in brainstem-type and cortical Lewy bodies**

Brainstem-type Lewy bodies, which consist of an eosinophilic core plus a surrounding pale halo (Fig. 2a), showed a ring-shaped XIAP-immunolabeling pattern, and the halos of these Lewy bodies were intensely immunostained (Fig. 2b). Some remaining neurons contained two or more brainstem-type Lewy bodies (Fig. 2c), and the marginal rims of these Lewy bodies were strongly immunoreactive for XIAP (Fig. 2d).

Cortical Lewy bodies, which are poorly-defined, eosinophilic structures without a clear halo (Fig. 2e and g), were intensely immunostained for XIAP (Fig. 2f and h). XIAP

immunoreactivity was localized over the entire bodies of these Lewy bodies (Fig. 2f and h).

### **Double-labeling immunohistochemistry for HtrA2/Omi and XIAP**

The double-immunostained sections of the midbrain and upper pons from patients with Parkinson's disease revealed the immunohistochemical co-localization of HtrA2/Omi and XIAP in brainstem-type Lewy bodies (Fig. 3a-c). The co-immunoexpression of both proteins was detected in the halos of these Lewy bodies (Fig. 3a-c). The average percentages of double-immunolabeled brainstem-type Lewy bodies in the substantia nigra and locus ceruleus were 67.5% and 58.8%, respectively.

The double-immunostained sections of the frontal and temporal cortices from patients with dementia with Lewy bodies revealed the immunohistochemical co-localization of HtrA2/Omi and XIAP in cortical Lewy bodies (Fig. 3d-f). The co-immunoexpression of both proteins was detected in the entire bodies of these Lewy bodies (Fig. 3d-f). The average percentages of double-immunolabeled cortical Lewy bodies in the cingulate and parahippocampal cortices were 56.6% and 68.4%, respectively.

### **XIAP immunoreactivity in brains with Alzheimer's disease**

In the frontal and temporal cortices from patients with Alzheimer's disease, many XIAP-immunoreactive senile plaques were distributed (Fig. 4a), and strong XIAP immunoreactivity was observed in the cores of some senile plaques (Fig. 4b). These immunostaining patterns were similar to those of HtrA2/Omi in brains with Alzheimer's disease [12], and our results indicate that neither XIAP nor HtrA2/Omi accumulate specifically in brainstem-type and cortical Lewy bodies.

### **Discussion**

Strauss and colleagues initially reported missense mutations in the gene encoding HtrA2/Omi in some patients with sporadic Parkinson's disease [13]. They also described the immunohistochemical localization of XIAP to brainstem-type Lewy bodies in autopsied brains with Parkinson's disease, but the immunohistochemical data on XIAP were not shown in their paper [13]. In accordance with their results, we observed that brainstem-type Lewy bodies were intensely immunostained for XIAP. In addition to brainstem-type Lewy bodies, we also observed that cortical Lewy bodies were strongly immunoreactive for XIAP. Furthermore, we observed the co-localization of XIAP and HtrA2/Omi immunoreactivities in the brainstem-type and cortical Lewy

bodies. To our best knowledge, this is the first report that documents the immunohistochemical localization of XIAP not only in brainstem-type Lewy bodies, but also in cortical Lewy bodies, and the co-immunoexpression of XIAP and HtrA2/Omi in both types of Lewy bodies.

In the present study, we demonstrated that XIAP immunoreactivity was localized to the halos of brainstem-type Lewy bodies and to the entire bodies of cortical Lewy bodies. These immunolabeling patterns were similar to those of HtrA2/Omi in brains with Parkinson's disease or dementia with Lewy bodies [11]. Our double-immunohistochemical studies revealed the co-localization of XIAP and HtrA2/Omi immunoreactivities in both types of Lewy bodies. HtrA2/Omi is released from the mitochondrial intermembrane space into the cytosol upon receiving various apoptotic stimuli, and this released HtrA2/Omi induces apoptotic cell death by binding to various IAPs, including XIAP, and blocking their caspase-inhibitory activities [7-10]. Since the interaction between HtrA2/Omi and XIAP occurs in the extra-mitochondrial areas, intra-mitochondrial HtrA2/Omi is not able to interact with XIAP in normal appearing neurons, but our results suggest that HtrA2/Omi may be released from the mitochondria into the cytosol in Lewy body-containing neurons, and that this extra-mitochondrial

HtrA2/Omi may make a partial contribution to neuronal cell death in brains with Parkinson's disease and dementia with Lewy bodies.

The striatal administration of adenovirus-mediated XIAP and glial cell line-derived neurotrophic factor was demonstrated to have synergistic effects for rescuing midbrain dopaminergic neurons from cell death, and for maintaining the nigrostriatal terminals in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse models of Parkinson's disease [14]. The neuronal overexpression of XIAP was also shown to attenuate MPTP-induced dopaminergic neuronal degeneration in the substantia nigra using XIAP transgenic mice under the control of the neuron-specific enolase promoter [15]. These data suggest that XIAP may play an important role in protecting nigral dopaminergic neurons in brains affected by Parkinson's disease. On the other hand, activated caspase-3 immunoreactivity was reported to be increased in the substantia nigra from patients with Parkinson's disease [16,17]. Using the TdT-mediated dUTP-biotin nick-end labeling (TUNEL) method, Mochizuki and coworkers showed that some dopaminergic neurons were TUNEL-positive in the substantia from patients with Parkinson's disease, and suggested that apoptosis may be associated with dopaminergic neuronal death in patients with Parkinson's disease [18]. In the present study, we observed the

localization of XIAP immunoreactivity in the brainstem-type Lewy bodies in the substantia nigra from patients with Parkinson's disease. Our findings suggest that the sequestration of XIAP into the brainstem-type Lewy bodies may suppress the anti-apoptotic function of XIAP, and that this dysfunction of XIAP may partially contribute to apoptotic cell death in the substantia nigra affected by Parkinson's disease.

## Conclusion

In this study, we demonstrated that XIAP immunoreactivity densely accumulated in the brainstem-type and cortical Lewy bodies in brains with Parkinson's disease or dementia with Lewy bodies, and our results suggest that XIAP may be partially associated with the pathogenesis of these Lewy body-related diseases.

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## Figure legends

**Figure 1.** XIAP immunoreactivity in the frontal cerebral cortex (a, b) and white matter (c, d) from normal subjects. Both neurons (a, b) and glial cells (c, d) were immunostained for XIAP. XIAP immunoreactivity in the substantia nigra from control (e) and Parkinson's disease (f) cases. The somata and processes of the pigmented neurons were immunoreactive for XIAP in both cases (e, f). Scale bars = 50  $\mu$ m (a, c), 20  $\mu$ m (b, d) and 100  $\mu$ m (e, f).

**Figure 2.** Midbrain sections stained with H&E (a, c) and immunostained for XIAP (b, d). Brainstem-type Lewy bodies are eosinophilic structures with a central core and a peripheral halo (a, c), and the halos of these Lewy bodies were intensely immunostained for XIAP (b, d). As indicated by the arrows, the Lewy bodies in (a) and (c) were identical to those in (b) and (d), respectively. Frontal cortical sections stained with H&E (e, g) and immunostained for XIAP (f, h). Cortical Lewy bodies, which are homogeneously eosinophilic structures without a clear halo (e, g), were intensely immunostained for XIAP (f, h). As indicated by the arrows, the Lewy bodies in (e) and (g) were identical to those in (f) and (h), respectively. Scale bars = 20  $\mu$ m (a-h).

**Figure 3.** Double-immunofluorescence staining for HtrA2/Omi (a) and XIAP (b) in the substantia nigra from patients with Parkinson's disease. The merged images showed that the HtrA2/Omi and XIAP immunoreactivities were co-localized to the brainstem-type Lewy bodies (c). The arrows in (a) to (c) indicate the same Lewy bodies. Double-immunofluorescence staining for HtrA2/Omi (d) and XIAP (e) in the frontal cortex from patients with dementia with Lewy bodies. The merged images showed that the HtrA2/Omi and XIAP immunoreactivities were co-localized to the cortical Lewy bodies (f). The arrows in (d) to (f) indicate the same Lewy bodies. Scale bars = 20  $\mu\text{m}$  (a-f).

**Figure 4.** XIAP immunoreactivity in the frontal cortex from patients with Alzheimer's disease. Many senile plaques were immunoreactive for XIAP (a), and strong XIAP immunoreactivity was found in the cores of some senile plaques (b, arrow). Scale bars = 40  $\mu\text{m}$  (a) and 20  $\mu\text{m}$  (b).